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Kitchen Refuse : a Novel Substrate for L(+) – Lactic Acid Production by *Rhizopus oryzae* in Submerged Fermentation

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Lactic acid fermentation from kitchen refuse by *Rhizopus oryzae* KPS 106 was studied in this work as a biological method of converting food waste into value added-products. Our aim was to achieve high-concentration and high-purity L(+)–lactic acid production. Several pretreatment methods to modify kitchen refuse as a medium were adopted: saccharification, natural precipitation, freezing and thawing technique, centrifugation and chromatography. The main component of sugar in saccharified kitchen refuse (SKR) was glucose (98.5% of total sugar). The most efficient technique for removing suspended solids (SS) in SKR was a freezing and thawing technique, by which approximately 55% of SS was eliminated. Kitchen refuse syrup (KRS) as a separated sugar from SKR by ion-exclusion chromatography could be used as a substrate for lactic acid fermentation by *R. oryzae* KPS 106 in a submerged culture. Addition of some inorganic minerals to KRS was effective in enhancing sugar consumption and lactic acid production. The sugar in KRS medium was assimilated almost as well as glucose in synthetic medium. The lactic acid yield from the KRS and glucose media were $63.9 \pm 1.8\%$ and $73.3 \pm 2.9\%$ (w/w) (based on initial total sugar concentration), respectively after 72 h of rotary shaking at 35°C and a rate of 100 rpm. L(+)–lactic acid produced from KRS medium was $98.7 \pm 0.3\%$ in purity. From these results, kitchen refuse was demonstrated to be a promising renewable substrate for lactic acid fermentation, a use that would be new strategy for converting food waste into value-added products.

Key words: Kitchen refuse, Lactic acid, *Rhizopus oryzae*

1. Introduction

Food waste in Japan was around 20 million tons per year in 1996. Most of it was simply dumped in landfills or incinerated. Only 9% of food waste was recycled as animal feed or fertilizer [1]. Therefore, new concepts for converting food waste into value-added products are expected.

L(+)–lactic acid is widely used in food, pharmaceutical, cosmetic and chemical industries. L(+)–lactic acid is also a key component for poly-lactic acid (PLA), with biodegradable, biocompatible and resorbable characteristics. The most effective way for L(+)–lactic acid synthesis is through biosynthesis rather than chemical processes [2]. Production of high-concentration and high-purity L(+)–lactic acid from renewable resources would be much more attractive [3].

To date, L(+)–lactic acid is produced commercially by fermentation of sugars with homofermentative lactic acid

bacteria or petrochemical processes that produce racemic mixtures of lactic acid. Although the carbon sources for lactic acid production by bacteria are limited, *Rhizopus oryzae* can produce high-purity lactic acid [4] in high quantities from various carbon sources such as glucose [5], steam-wood hydrolysate [6], corn starch [7], carrot processing solid waste [8], potato pulp [9] and corn cobs [10].

Kitchen refuse is rich in organic material, with high levels of carbohydrates, proteins and some mineral salts. Conversion of food waste into various products such as fertilizer [11], methane [12] and animal feed [13] has been developed using fermentation techniques. However, little research has been done on the utilization of kitchen refuse for lactic acid production by *R. oryzae*. Thus, the objective of this research was to study the applicability of kitchen refuse with pretreatment techniques (saccharification, precipitation, freezing-thawing and chromatography) as a medium for the production of lactic acid by *R. oryzae* KPS 106 in submerged culture under shaking flask conditions. The kinetics of lactic acid fermentation from kitchen refuse by *R. oryzae* were compared with those from

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other substrates. The optical purity of the lactic acid was also analyzed.

2. Materials and Methods

2.1 Pretreatment of kitchen refuse

2.1.1 Saccharification method

The kitchen refuse was collected from Yahata Royal Hotel (Kitakyushu, Fukuoka, Japan) and treated by the following procedures, illustrated in Fig.1. Each item of kitchen refuse was separated and weighed before saccharification. The main items of kitchen refuse were carbohydrates, proteins and vegetables mixed with fruits. The percentage of each item for saccharification was 43%, 19% and 38% (w/w), respectively. The composition of kitchen refuse for this experiment is shown in Table 1. Saccharification was carried out for 6 h in a rotating drum-shaped reactor by adding 300 ppm glucoamylase (Nagase Enzyme, Japan) and 10% (w/w) of water in a water bath at a temperature of 50°C and rotation rate of 50 rpm. Solids that could not completely hydrolyzed were removed by filtration through a wire mesh (pore size 6 × 6 mm). The liquor obtained was called saccharified

Table 1 Composition of kitchen refuse for saccharification.

Kitchen waste fraction	Percentage (w/w, wet weight)	Total of percentage (w/w, wet weight)
Carbohydrates group		
Spaghetti	4.8	43
Bread	3.0	
Maccaroni	1.4	
Rice	33.8	
Proteins group		
Fried egg	2.5	19
Salmon	3.5	
Bacon	1.0	
Sausage	2.0	
Threadsail	5.6	
Seabream	1.4	
Bonite	3.0	
Vegetables and fruits group		
Broccoli	7.5	38
Cucumber	1.1	
Scallion	0.2	
Long white radish	2.0	
Cabbage	1.8	
Carrot	2.4	
Onion	1.5	
Lettuce	11.0	
Japanese leek	0.5	
Enoki mushroom	0.7	
Pear	6.3	
Grape	1.0	
Lemon	0.9	
Orange	1.1

Fig. 1 Pretreatment of kitchen refuse as lactic acid fermentation medium.

kitchen refuse (SKR). Particles of SKR were precipitated by keeping the mixture static at a temperature 4°C overnight. The obtainable supernatant was further cleared by the freezing and thawing method.

2.1.2 Freezing and thawing method

Suspended Solids (SS) in SKR were removed by following the method reported by Phang *et al.* [14]. About 300 mL of SKR was placed in an ice cube tray to produce ice cubes by freezing at −30°C for more than 12 h in a home freezer. The frozen mass was thawed on a wire mesh (pore size 2 mm × 2 mm) at room temperature. The melted drips were collected and centrifuged, first at 4,000 rpm for 10 min. The supernatant from this step was used for fermentation and denoted as FKR solution. A second round of centrifugation at 3,000 rpm for 5 min was performed; the supernatant was subjected to ion-exclusion chromatography for the separation of sugars. SS content was measured by APHA Standard Methods [15].

2.1.3 Ion-exclusion chromatography

A plastic chromatography column 2.5 cm in diameter and 45.0 cm in length was packed with Amberlite CR–

1310Na (ORGANO, Japan) resin. The Amberlite CR-1310Na was equilibrated with 500 mL of 2 M NaCl; then 20 mL of sample was applied to the column. Elution was performed with distilled water at a flow rate of 3 mL/min. Fractions (3 mL) were collected, and the concentration profiles of the elution curves were determined in order to identify the glucose fractions. Fractions which showed high concentration of glucose without contamination by any metal ion were collected. Metal ion contamination was checked by the electrical conductivity meter CM21P (TOA, Japan). Kitchen refuse syrup (KRS) was obtained after concentration by rotary vacuum evaporation (EYELA, Japan) at 40°C and 50 rpm.

2.2 Fermentation

2.2.1 Microorganism and inoculum

R. oryzae KPS 106, L(+)–lactic acid producing fungi was used throughout this study, under slow shaking speed. This strain has been mutated by UV irradiation [16] and was obtained from Kasetsart University (Bangkok, Thailand). The fungi were maintained in a medium containing Difco potato-dextrose agar (PDA), and transferred to fresh slants every week. Spores were produced on PDA after 8 days of culture at 35°C. They were collected in sterile distilled water containing 0.1% Tween 80 by platinum loop and filtrated through sterile cotton. A spore suspension with 10^7 spores/mL was used as an inoculum. The spore number in suspension was counted using a hemocytometer.

2.2.2 Culture medium

The composition [g/L] of the medium was: carbon source, 100 with the following mineral supplements (S): ammonium sulfate, 3.20; potassium dihydrogenphosphate, 0.30; magnesium sulfate, 0.25 and zinc sulfate 0.04.

FKR, KRS and glucose were used as carbon sources and prepared as culture media with mineral supplements, denoted as FKR+S, KRS+S and glucose medium. The purpose of the comparison was to investigate the effects of medium composition from kitchen refuse on lactic acid fermentation. Pure FKR and KRS were also used as media and described as FKR and KRS.

To evaluate the effect of carbohydrates on lactic acid production, sucrose, galactose, glucose, saccharose, dextrin, cassava starch, potato starch and kitchen refuse were also tested as carbon sources. The pH values of the media were not controlled. All media were sterilized at 121°C for 15 min.

2.2.3 Culture method

A 300-mL Erlenmeyer flask containing 50 mL of medium

was inoculated with 1 mL of spore suspension and incubated in a rotary shaker at 35°C and 100 rpm. After 24 h, 2.5 g of sterilized calcium carbonate was added to each flask as pH neutralizer and cultivation carried out until 72 h.

2.3 Analytical methods

The mycelium dry weight was determined at the end of the experiment by filtering the entire culture broth. The mycelia were washed twice by 2 M HCl and distilled water, respectively. After that they were dried at 105°C for 24 h and the biomass was weighed. Concentration of substrates and the fermentation products in the fermentation broth were analyzed. The concentration of lactic acid and other organic acids were determined by HPLC, using a Shim-pack SCR-102H column (Shimazu, Japan). The eluent was 951 mg/mL of *p*-toluenesulfonic acid at a flow rate of 0.8 mL/min. The column temperature was maintained at 40°C and a refractive index (RI) detector was used. Lactic acid purity was analyzed by a L-lactic acid test kit (Boehringer, Germany). Glucose was determined by glucose oxydase and peroxydase enzyme (Glucose test kit, Toyobo, Japan). Total sugar was measured by the phenol sulfuric acid method [17]. The concentration of ethanol was determined by Boehringer Mannheim enzymatic bioanalysis kits (Cat. No. 0176290, Germany).

3. Results

3.1 Characteristics of kitchen refuse

Table 2 shows the characteristics of kitchen refuse after each pretreatment. SS removal from liquefied kitchen refuse after each pretreatment is clearly observed in Fig. 2. After saccharification of kitchen refuse by glucoamylase enzyme, no oligosaccharides were accumulated in SKR. The SKR contained with 98.5% glucose in total sugar and 98.9 g/L of SS. First, 14.6% of SS was eliminated by precipitation. A rather large amount of SS was removed by this

Table 2 Effect of pretreatment method on characteristics of kitchen refuse.

Pretreatment method	Total sugar remained [%]	Glucose remained [%]	Suspended solids remained [%]
Saccharification	100	100	100
Precipitation	99.8	99.2	85.4
Freezing and thawing	98.3	94.4	30.0
Centrifugation at 4,000 rpm for 10 min	93.0	92.5	2.7
Centrifugation at 3,000 rpm for 5 min	90.9	90.5	0.2
Ion-exclusion chromatography	56.1	55.9	0

Fig. 2 Photograph of kitchen refuse liquid at each stage of pretreatment.

- A. Saccharified kitchen refuse (SKR)
- B. Thawed solution of freezing and thawing technique
- C. Supernatant after centrifugation (FKR)
- D. Eluent from ion-exclusion chromatography
- E. Kitchen refuse syrup (KRS)

step. After that, we successfully removed 55.3% of SS by the freezing and thawing technique, but the remaining SS was still high (30.0%). The cloudy thawed solution including some lipids is shown in Fig. 2B. Consequently, 97.3% of SS removal was achieved after centrifugation at 4,000 rpm for 10 min. Finally, a clear supernatant with only 0.2 g/L of SS was obtained after the 2nd centrifugation at 3,000 rpm for 5 min. Throughout the series of pretreatment process, the remained glucose concentration in the solution was 55.9%.

3.2 Comparison of lactic acid fermentation from glucose and kitchen refuse by *R. oryzae* KPS 106

Comparison of lactic acid fermentation from kitchen refuse and glucose by *R. oryzae* is shown in Table 3. The major products produced by *R. oryzae* from kitchen refuse and glucose were lactic acid and ethanol. Small amounts of by-products (succinic acid, formic acid, fumaric acid and acetic acid) were detected. About 6 g/L of biomass and 20% (w/w) yield of ethanol were obtained from FKR, FKR+S,

KRS and KRS+S after 72 h. In the case using glucose, only 3.2±0.2 g/L of biomass and 7.7±2.0% (w/w) of ethanol yield were produced. Biomass and ethanol yields from the kitchen refuse medium were two times higher than those from glucose. Lactic acid yield from FKR and FKR+S was rather low when compared with that of KRS and KRS+S. In the case using KRS as substrate, lactic acid yield was increased and made close to that of glucose (from 51.0±1.5% to 63.9±1.8% (w/w)) by adding inorganic minerals (S) ((NH₄)₂SO₄, KH₂PO₄, MgSO₄·7H₂O and ZnSO₄·7H₂O). This phenomenon was observed when both FKR and KRS were used as substrates. The highest lactic acid yield was 63.9±1.8% (w/w) from KRS+S. The lowest lactic acid yield was 12.3±1.8% (w/w) from the medium containing only FKR. Based on these results, KRS+S was used for the subsequent experiment and denoted as KRS medium.

3.3 Effect of various carbohydrates on lactic acid production by *R. oryzae* KPS 106

The effect of various carbohydrates on lactic acid production by *R. oryzae* KPS 106 was investigated. Sucrose, galactose, glucose, saccharose, dextrin, cassava starch, potato starch and KRS were used as carbon sources in the medium, as shown in Table 4. The highest lactic acid yield of 73.3% (w/w) was obtained from glucose. The next highest lactic acid yield was from cassava starch and KRS medium with 64.6 and 63.4% (w/w), respectively. Distinctive differences in ethanol yield (21.2% (w/w)) and biomass (5.9 g/L) were observed when using KRS medium, while only 6–8 % (w/w) ethanol yield and about 2–4 g/L of biomass were obtained from other media.

3.4 Kinetics of lactic acid fermentation by *R. oryzae* KPS 106

Fig. 3 shows the kinetics of lactic acid fermentation by *R. oryzae* KPS 106. Glucose and KRS medium showed the

Table 3 Comparison of lactic acid production from kitchen refuse and glucose by *R. oryzae* KPS 106.

Medium	Dry weight [g/l]	Sugar consumption [%]	Succinic acid yield [%]	Lactic acid yield [%]	Formic acid yield [%]	Fumaric acid yield [%]	Acetic acid yield [%]	Ethanol yield [%]
FKR	5.8±0.1	53.2±2.3	0	12.3±1.8	0	0.1±0.0	0.5±0.2	21.0±1.5
FKR+S	5.9±0.2	99.5±2.4	0	16.5±9.6	0	0	0.5±0.2	18.4±2.9
KRS	6.2±0.2	59.3±1.9	0	51.0±1.5	0	0.0	0.4±0.1	20.9±1.3
KRS+S	5.9±1.3	99.6±0.4	0	63.9±1.8	0	0.1±0	0.4±0.1	21.2±2.6
Glucose	3.2±0.2	100±0	0	73.3±2.9	0	0	0.6±0.3	7.7±2.0

Means ± standard deviations for n=5
% Yield based on initial total sugar concentration
FKR is saccharified kitchen refuse after 1-time centrifugation
KRS is kitchen refuse syrup
S is inorganic minerals

Table 4 Effect of various carbohydrates on the production of lactic acid by *R. oryzae* KPS 106.

Carbohydrate [100g/l]	Succinic acid yield [%]	Lactic acid yield [%]	Formic acid yield [%]	Fumaric acid yield [%]	Acetic acid yield [%]	Ethanol yield [%]	Dry weight [g/L]
Sucrose	0	53.8	0	0	0.5	6.5	3.1
Fructose	0	54.1	0	0.1	0.5	7.3	4.2
Galactose	0	57.3	0	0.1	0.5	5.1	3.3
Glucose	0	73.3	0	0	0.6	7.7	3.2
Saccharose	0	29.1	0	0.1	0.2	5.0	2.1
Dextrin	0	45.0	0	0.1	0.4	5.9	2.5
Cassava starch	0.1	64.6	0	0.1	0.7	7.0	4.3
Potato starch	0	50.6	0	0.1	0.5	6.2	3.8
KRS	0	63.4	0	0.1	0.4	21.2	5.9

% Yield based on initial total sugar concentration

Cassava starch and potato starch were liquefied with enzyme amylase (Sigma, Lot 67H1563)

Fig.3 Kinetics of lactic acid production from glucose (A) and KRS (B) medium by *R. oryzae* KPS 106 (■ Sugar, ● Lactic acid, ▲ Ethanol).

same trend of lactic acid production and sugar consumption. Most of the sugar in both media was completely consumed. Two modes of cell growth and lactic acid production phases were observed. The production of lactic acid during the growth phase was relatively low from both carbon sources. The rate of sugar consumption and lactic acid production were found not to be influenced by the carbon source. In KRS medium, 21.6 g/L of ethanol was detected. Under the same conditions, only 9 g/L of ethanol was generated from the glucose medium. At 72 h, 71.4 g/L (72.5% (w/w)) and 63.1 g/L (63.9% (w/w)) of lactic acid were accumulated in glucose and KRS media, respectively.

Furthermore, $98.7 \pm 0.3\%$ of the lactic acid produced from KRS medium by the *R. oryzae* KPS 106 was the L(+)-isomer (data not shown).

4. Discussion

After saccharification of kitchen refuse, SS in SKR were in a colloidal form that was insufficient of free air space to allow for oxygen transfer. Under these oxygen-limited

conditions, lactic acid fermentation by *R. oryzae* resulted in partial conversion to ethanol, fumaric acid or other acids, as determined by Skory *et al.* [18]. Consequently, it is difficult to achieve high lactic acid yield when SKR was applied directly. To overcome this problem, SS removal by various pretreatments to SKR were examined. 55.3% of SS was successfully removed by freezing and thawing, as shown in Table 2. Ion-exclusion chromatography was conducted for separation of mineral salts or other components that may affect lactic acid production by *R. oryzae*. As reported by Zhou *et al.* [19], concentrations of nitrogen source, phosphate, and metal ion contents have a strong effect on lactic acid fermentation and growth of *R. oryzae* when glucose is used as carbon source. Their research supports our results that little lactic acid was produced from FKR while lactic acid yield and sugar consumption increased using KRS form which metal ions had been removed by ion-exclusion chromatography. This indicates that the ingredients in kitchen refuse have a significant effect on lactic acid production and sugar consumption of *R. oryzae* KPS 106.

The major products of *R. oryzae* KPS 106 in submerged culture with carbohydrate or kitchen refuse were lactic acid and ethanol. Furthermore, small amounts of succinic acid, formic acid, fumaric acid and acetic acid were detected. The product yields were strongly influenced by the medium composition, as shown in Table 3. According to Sun *et al.* [20], sugar consumption and lactic acid production were high when the medium contained inorganic salts. And normally, lactic acid fermentation medium contains some inorganic minerals: $(\text{NH}_4)_2\text{SO}_4$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, KH_2PO_4 and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ [6,7,20]. Therefore some inorganic supplements were required to improve lactic acid yield and sugar consumption when using kitchen refuse

Table 5 Summary of lactic acid production by *R. oryzae* from various recycled wastes.

Recycle waste	Culture	Lactic acid [g/L]	Lactic acid yield [%]	References
Carrot waste	Submerged	24.00	0.6	Garg and Hang (1995)
Hemicellulosic hydrolysate	Submerged	12.00	0.3	Woiciechowski <i>et al.</i> , (1999)
Potato pulp	Solid state	10.3 mg/g fresh matter	–	Oda <i>et al.</i> , (2002)
Potato pulp	Solid state	10.2 mg/g fresh matter	–	Saito <i>et al.</i> , (2003)
Corn cobs	Solid state	299.4±6.8 g/kg dry matter of corn cobs	–	Ruengruglikit and Hang (2003)
Waste OA paper	Submerged	49.1	0.6	Park <i>et al.</i> , (2003)
Kitchen refused	Submerged	64.8	0.6	This work

rich in organic matter content [21] as medium. The highest lactic acid yield was obtained from KRS+S. From this result, KRS+S comprised the optimal composition of kitchen refuse to achieve high lactic acid yield by *R. oryzae* KPS 106.

As can be seen from the Tables 3 and 4, kitchen refuse is a good potential substrate for lactic acid fermentation compared with other carbohydrates and organic wastes. Many researchers have been increasing their interest in lactic acid production by *R. oryzae* from various wastes. Corn cobs, carrot waste, potato pulp [22], steam-wood hydrolysate and waste OA paper [23] were studied by solid state or submerged culture. *R. oryzae* could produce lactic acid from various carbohydrates (mono-, di-, and poly-saccharides), because it synthesized extracellular amylolytic enzyme [24]. Hence, *R. oryzae* has been effective at generating lactic acid from various wastes. This study is the first report of the production of lactic acid by *R. oryzae* from multi and complex-component wastes such as kitchen refuse, and the lactic acid yield was greater than values reported by similar research on other media. Although the lactic acid yield from KRS by *R. oryzae* was lower than that from glucose as carbon source, the trend for lactic acid production from KRS was similar, as shown in Fig. 3. As for the cause of the disparity, dense mycelial growth of *R. oryzae* increased the viscosity of the KRS medium, causing the problem of limited oxygen transfer [25]. Therefore, lactic acid production was partially converted to production of ethanol or other by-products. This hypothesis is consistent with that fact that ethanol yield in the KRS medium was higher than that in the glucose medium.

The purity of lactic acid is important for the quality of final products such as poly-lactic acid (PLA). Therefore, selection of microorganisms producing high-purity L(+)-lactic acid is essential in this application. The purity of L(+)-lactic acid produced from KRS medium by *R. oryzae* KPS 106 was 98.7±0.3%. About 99% L(+)-lactic acid or

greater is usually recommended for polymerization [26].

These experiments proved that, high-concentration, high-purity L(+)-lactic acid can be produced from a low-cost fermentation medium when kitchen refuse is used for lactic acid fermentation by *R. oryzae* KPS 106.

5. Conclusions

The strong potential of kitchen refuse as renewable substrate for lactic acid production by *R. oryzae* KPS 106 was shown in this study. Kitchen refuse syrup (KRS) obtained after various pretreatments showed good assimilation, comparable to that of glucose. The highest lactic acid yield, 63.9±1.8% was obtained from KRS containing inorganic supplements, after 72 h in a rotary shaker at 35°C and 100 rpm. Moreover, 98.7±0.3% of the lactic acid produced was L(+)-isomer.

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◇◇◇◇ 和文要約 ◇◇◇◇

食品ゴミ： *R. oryzae* による L-乳酸発酵のための新しい基質

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食品ゴミを基質として、*Rhizopus oryzae* KPS106 株を用いた、高濃度ならびに高純度の L(+)-乳酸生産について検討した。食品ゴミに、種々の前処理（糖化、自然沈降、凍結融解、遠心分離、イオン排除クロマトグラフィー）を施して、培地として利用できるように調製した。食品ゴミ糖化液中、全糖比 98.51% はグルコースであった。食品ゴミ糖化液から懸濁固形分（SS）を除去するには、凍結融解法が有効であり、55 % の SS が除去された。その後、イオン排除クロマトグラフィーを経て、食品ゴミ糖化液から精製した糖を、*R. oryzae* KPS106 株による乳酸発酵の基質に用いた。生ゴミ培地中の糖を炭素源として、振とう培養（100 rpm, 35℃, 72 時間）

を行ったところ、初期全糖濃度に基づく乳酸収率は、食品ゴミ由来の培地で 63.9±1.8% であった。食品ゴミ糖化液から分離精製した糖は、合成培地のグルコースと同様に資化された。また、微量の無機成分の添加が、糖消費と乳酸生成を促進した。さらに、生成した L(+)-乳酸の光学純度は、98.7±1.8% であった。以上の結果から、食品ゴミは、生物学的変換により乳酸のような有価物を製造する有望な再生可能資源であることが示唆された。均質な廃棄物を原料とする *R. oryzae* による乳酸生産は、これまでも数多く報告されているが、食品ゴミの様に、雑多な成分の含まれるものを基質とする研究は、本報告が初めてである。